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(54) **METHOD OF USING A BIOSENSOR**

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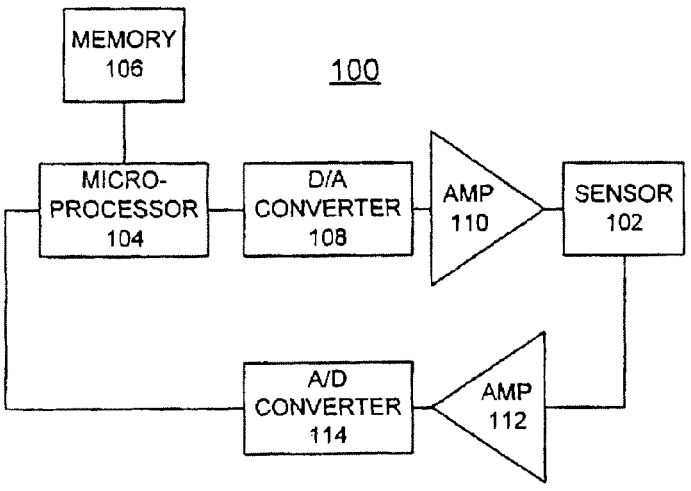
(57) **ABSTRACT**

A biosensor (**102**) for determining the presence or amount of a substance in a sample and methods of use of the biosensor (**102**) are provided. The biosensor (**102**) for receiving a user sample to be analyzed includes a mixture for electrochemical reaction with an analyte. The mixture includes an enzyme, a mediator and an oxidizable species as an internal reference.

26 Claims, 10 Drawing Sheets



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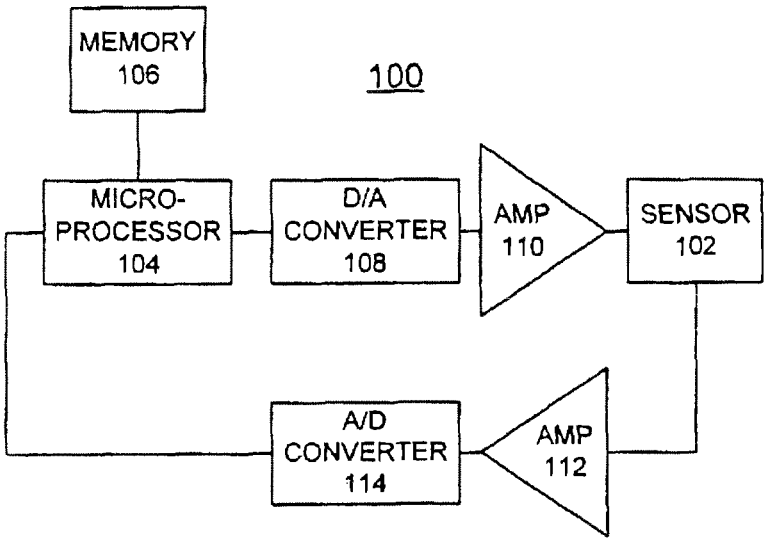

FIG. 1A

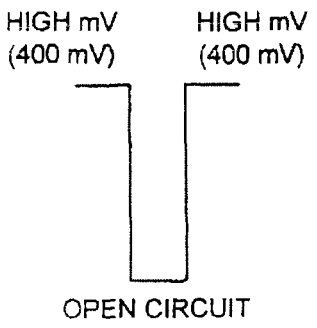

FIG. 1B

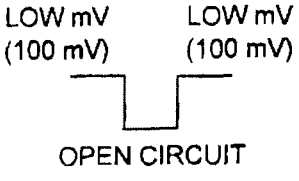

FIG. 1C

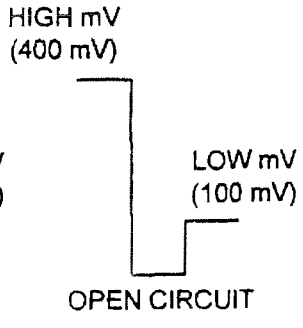

FIG. 1D

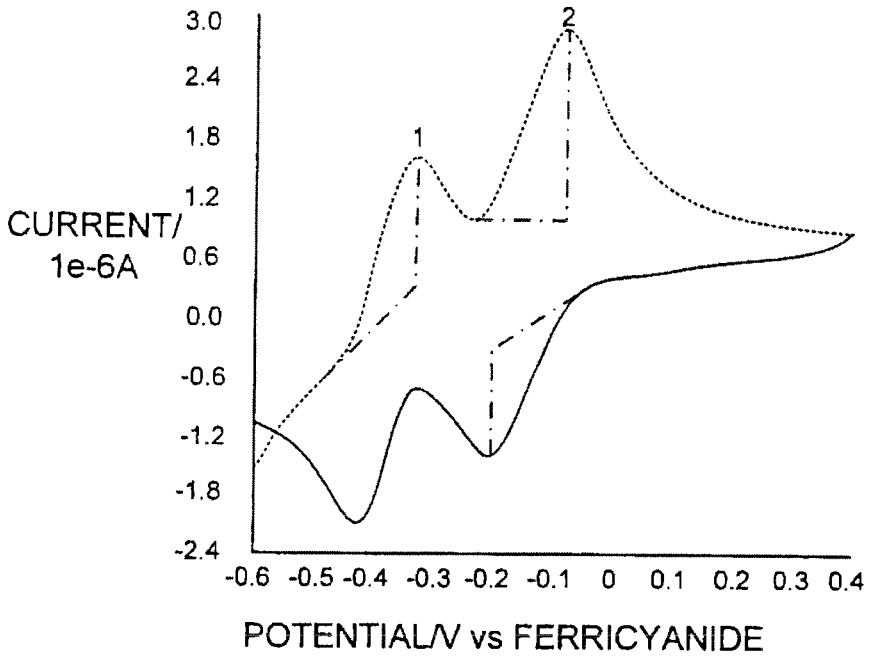
3.0
2.4
1.8
1.2
0.6
0.0
-0.6
-1.2
-1.8
-2.4
CURRENT/
1e-6A
1
2
-0.6 -0.5 -0.4 -0.3 -0.2 -0.1 0 0.1 0.2 0.3 0.4
POTENTIAL/V vs FERRICYANIDE

FIG. 2A

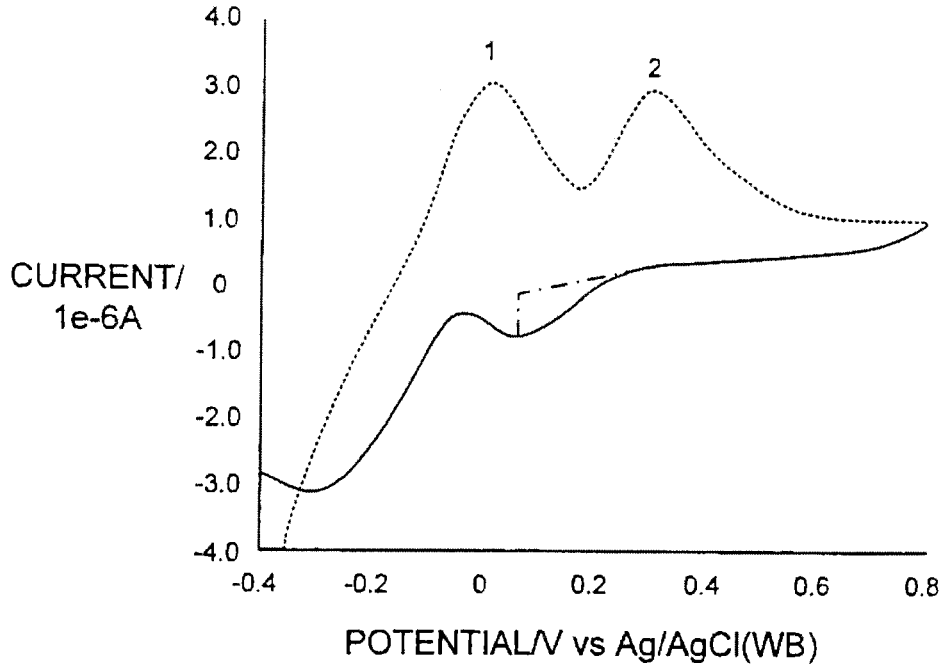
4.0
3.0
2.0
1.0
0
-1.0
-2.0
-3.0
-4.0
CURRENT/
1e-6A
1
2
-0.4
-0.2
0
0.2
0.4
0.6
0.8
POTENTIAL/V vs Ag/AgCl(WB)

FIG. 2B

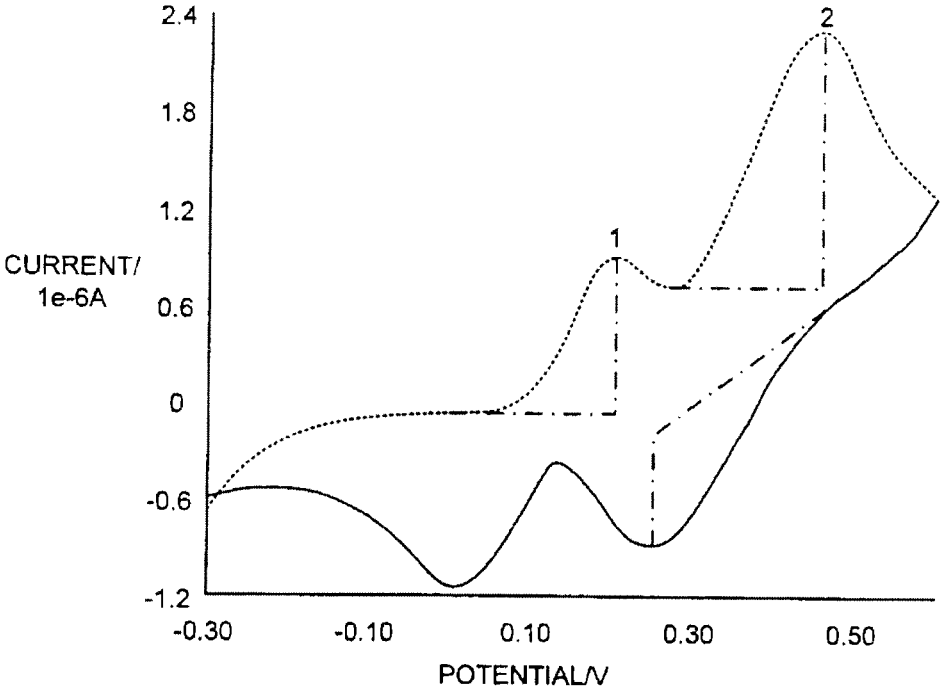
2.4
1.8
1.2
0.6
0
-0.6
-1.2
CURRENT/
1e-6A
1
2
-0.30
-0.10
0.10
0.30
0.50
POTENTIAL/V

FIG. 2C

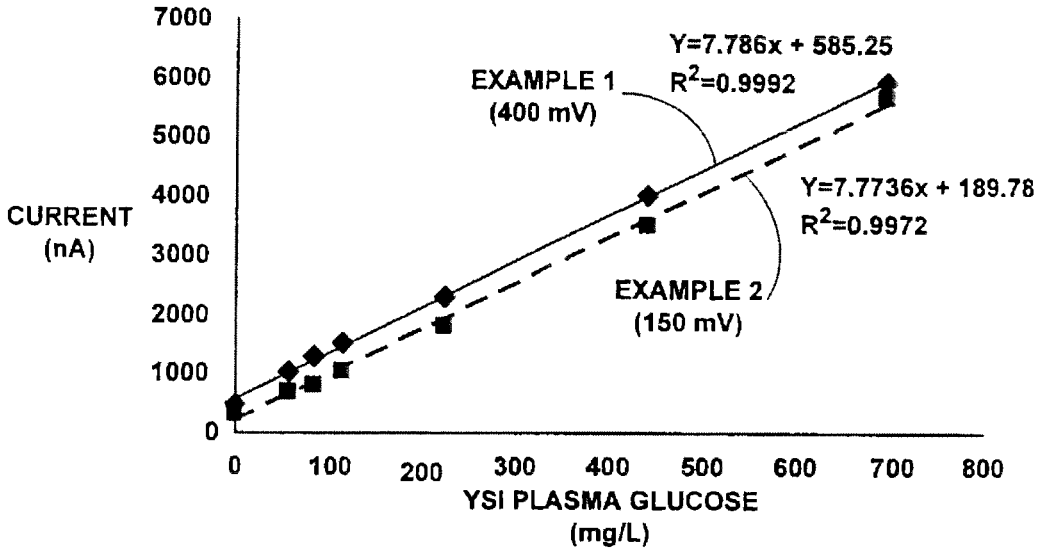
7000
6000
5000
4000
3000
2000
1000
0
CURRENT
(nA)
Y=7.786x + 585.25
R2=0.9992
EXAMPLE 1
(400 mV)
Y=7.7736x + 189.78
R2=0.9972
EXAMPLE 2
(150 mV)
0
100
200
300
400
500
600
700
800
YSI PLASMA GLUCOSE
(mg/L)

FIG. 3

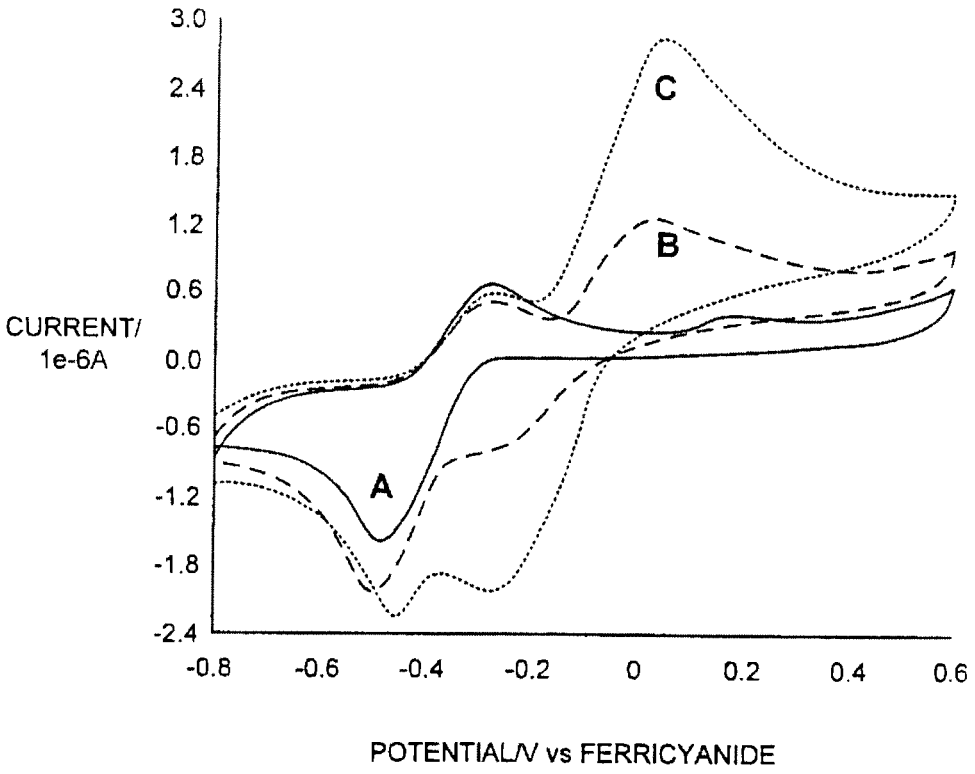
3.0
2.4
1.8
1.2
0.6
0.0
-0.6
-1.2
-1.8
-2.4
CURRENT/
1e-6A
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
A
B
C
POTENTIAL/V vs FERRICYANIDE

FIG. 4

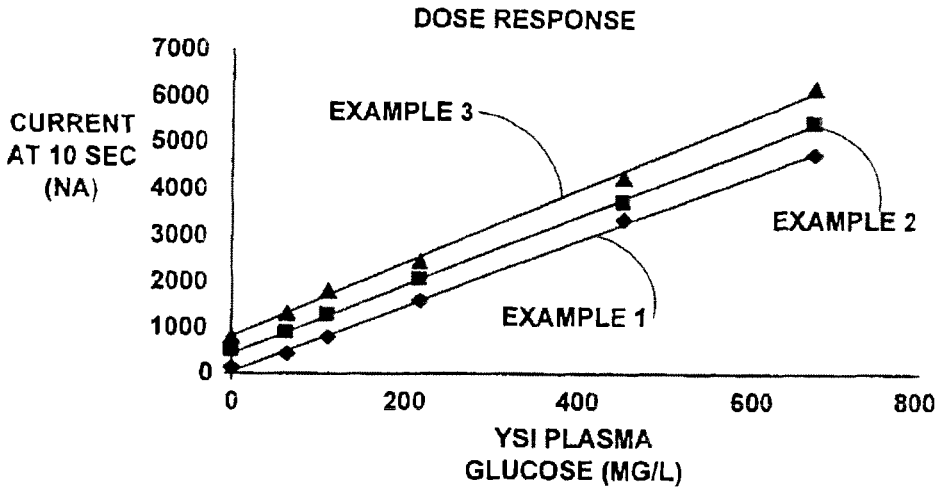
DOSE RESPONSE
CURRENT AT 10 SEC (NA)
7000
6000
5000
4000
3000
2000
1000
0
EXAMPLE 3
EXAMPLE 2
EXAMPLE 1
0
200
400
600
800
YSI PLASMA GLUCOSE (MG/L)

FIG. 5A

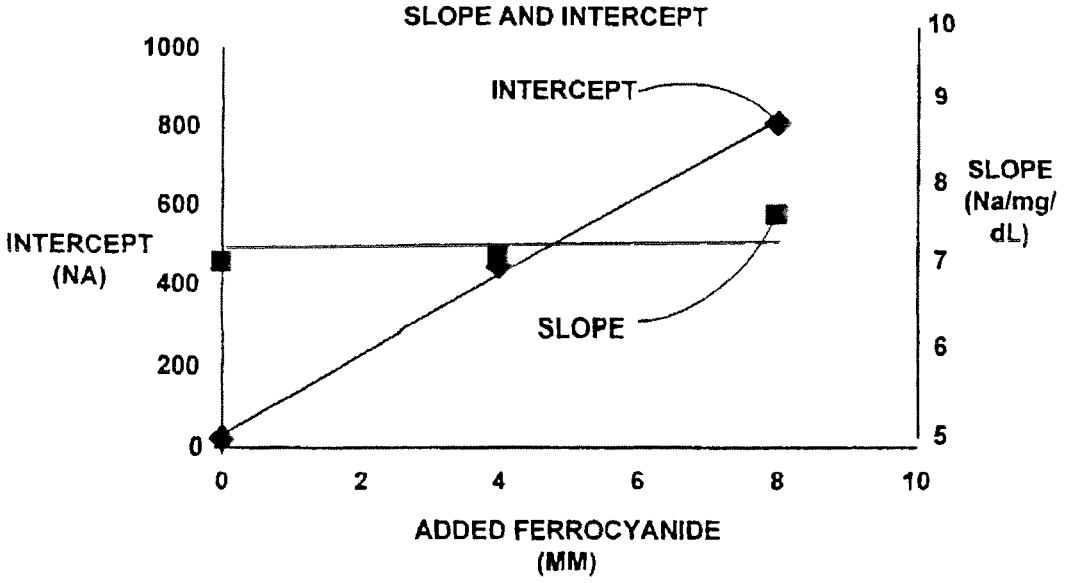
SLOPE AND INTERCEPT
INTERCEPT (NA)
1000
800
600
400
200
0
INTERCEPT
SLOPE
SLOPE (Na/mg/dL)
10
9
8
7
6
5
0
2
4
6
8
10
ADDED FERROCYANIDE (MM)

FIG. 5B

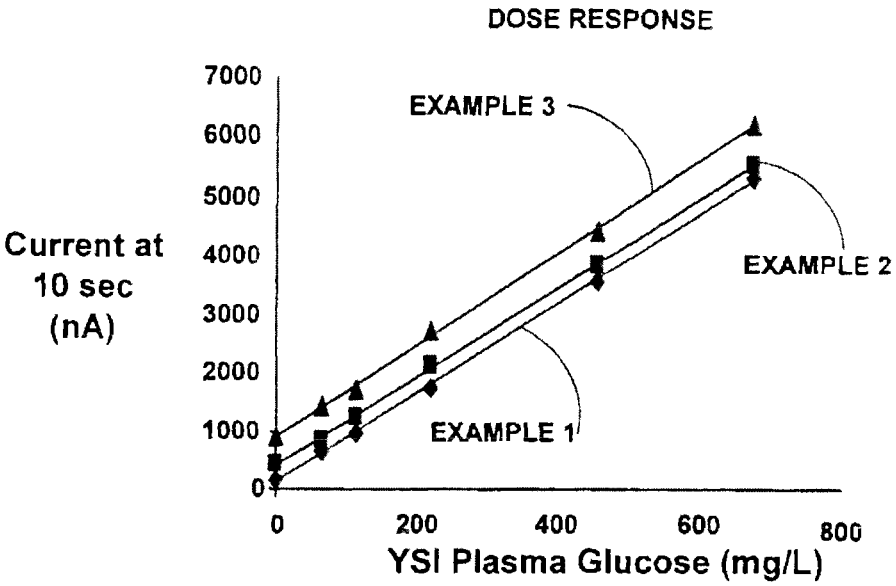
DOSE RESPONSE
Current at 10 sec (nA)
7000
6000
5000
4000
3000
2000
1000
0
EXAMPLE 3
EXAMPLE 2
EXAMPLE 1
0
200
400
600
800
YSI Plasma Glucose (mg/L)

FIG. 6A

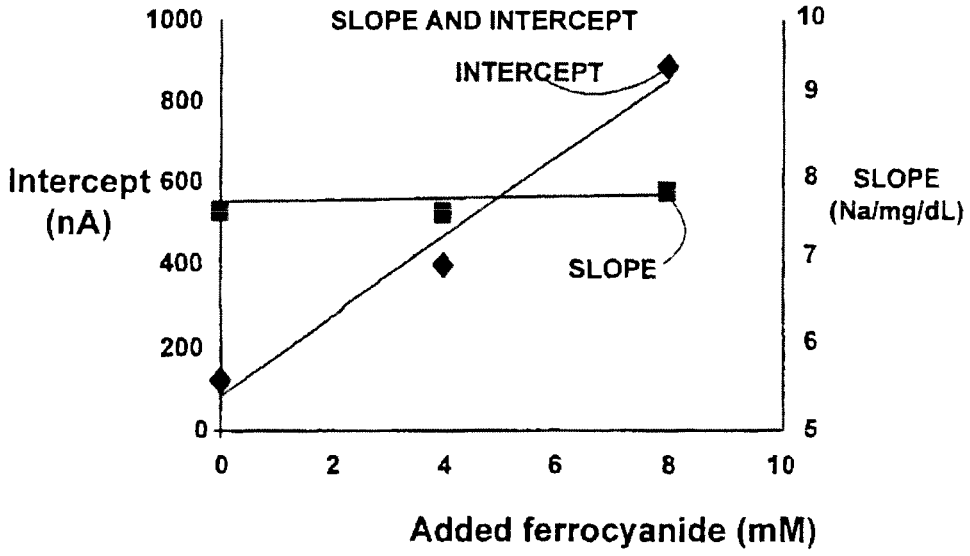
SLOPE AND INTERCEPT
Intercept (nA)
1000
800
600
400
200
0
INTERCEPT
SLOPE
10
9
8
7
6
5
SLOPE (Na/mg/dL)
0
2
4
6
8
10
Added ferrocyanide (mM)

FIG. 6B

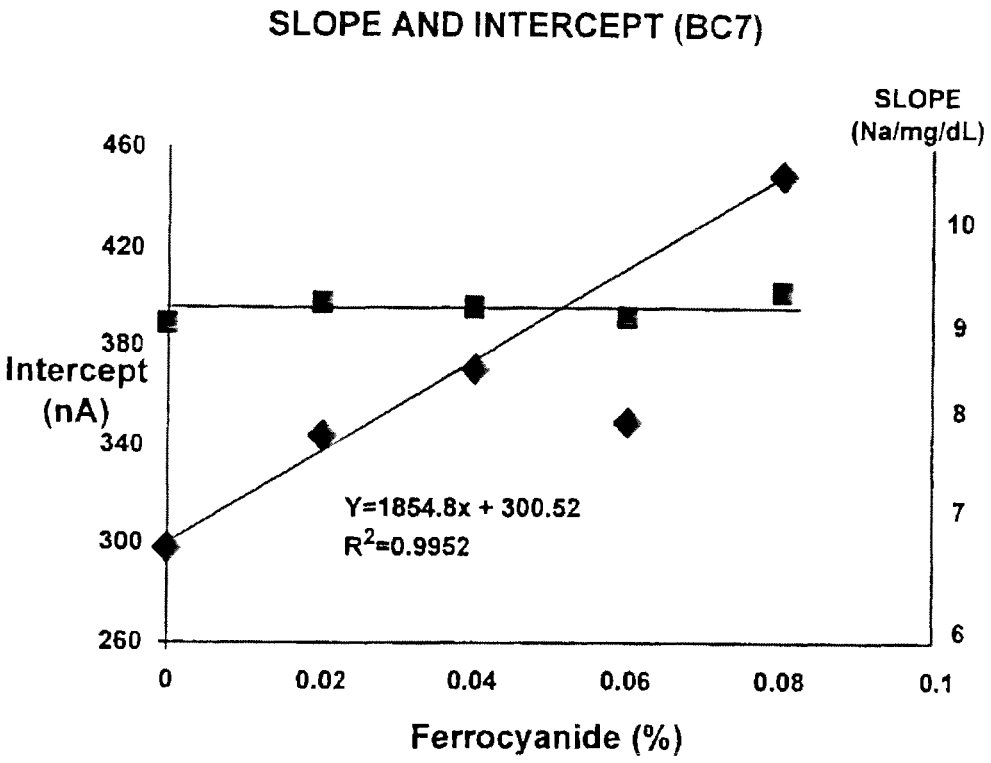
SLOPE AND INTERCEPT (BC7)
SLOPE
(Na/mg/dL)
460
420
380
340
300
260
Intercept
(nA)
10
9
8
7
6
Y=1854.8x + 300.52
R2=0.9952
0
0.02
0.04
0.06
0.08
0.1
Ferrocyanide (%)

FIG. 7

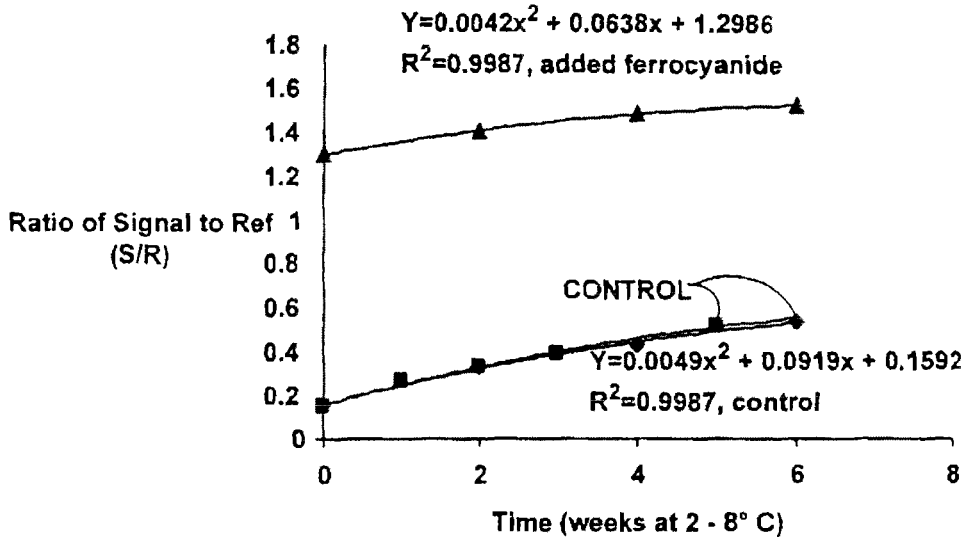
Y=0.0042x2 + 0.0638x + 1.2986
R2=0.9987, added ferrocyanide
Ratio of Signal to Ref (S/R)
CONTROL
Y=0.0049x2 + 0.0919x + 0.1592
R2=0.9987, control
Time (weeks at 2 - 8° C)

FIG. 8A

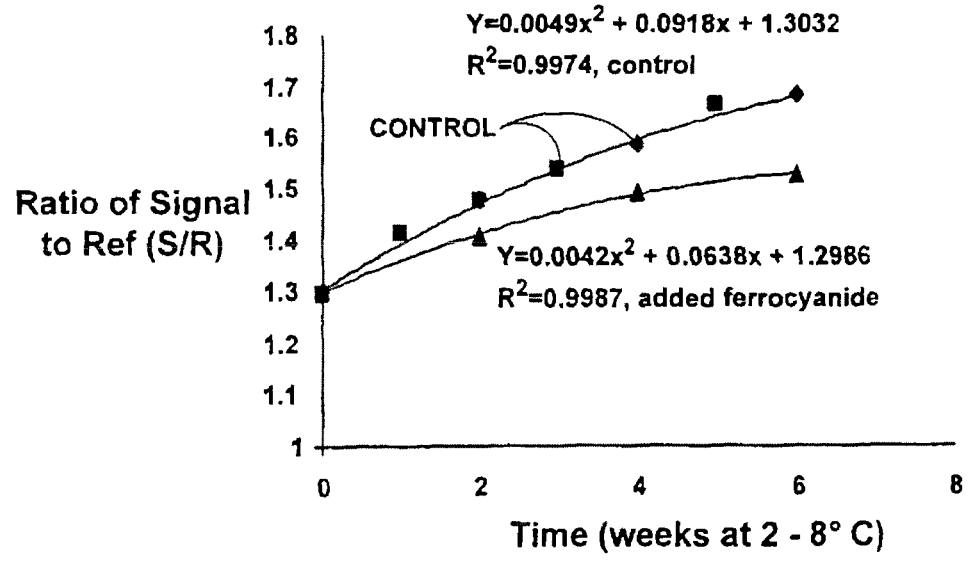
Y=0.0049x2 + 0.0918x + 1.3032
R2=0.9974, control
Ratio of Signal to Ref (S/R)
CONTROL
Y=0.0042x2 + 0.0638x + 1.2986
R2=0.9987, added ferrocyanide
Time (weeks at 2 - 8° C)

FIG. 8B

METHOD OF USING A BIOSENSOR

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a division of application Ser. No. 10/590,867 filed Aug. 24, 2006, which has been allowed; application Ser. No. 10/590,867 filed Aug. 24, 2006 is a nationalized application of Application No. PCT/US2005/03622 filed Feb. 4, 2005, which claims priority to Application No. 60/542,362 filed on Feb. 6, 2004, which are all incorporated by reference in their entireties.

FIELD OF THE INVENTION

The present invention generally relates to a biosensor, and, more particularly, to a new and improved biosensor, including an oxidizable species as an internal reference and methods of use of the biosensor, for determining the presence or amount of a substance in a sample.

DESCRIPTION OF THE PRIOR ART

The quantitative determination of analytes in body fluids is of great importance in the diagnoses and maintenance of certain physiological abnormalities. For example lactate, cholesterol and bilirubin should be monitored in certain individuals. In particular, the determination of glucose in body fluids is of great importance to diabetic individuals who must frequently check the level of glucose in their body fluids as a means of regulating the glucose intake in their diets. While the remainder of the disclosure herein will be directed towards the determination of glucose, it is to be understood that the new and improved sensor element and method of use of this invention can be used for the determination of other analytes upon selection of the appropriate enzyme.

Methods for determining analyte concentration in fluids can be based on the electrochemical reaction between the analyte and an enzyme specific to the analyte and a mediator which maintains the enzyme in its initial oxidation state. Suitable redox enzymes include oxidases, dehydrogenases, catalase and peroxidase. For example, in the case where glucose is the analyte, the reaction with glucose oxidase and oxygen is represented by equation:

(A)

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase (GO)}} \text{gluconolactone} + H_2O_2$$

In the initial step of the reaction represented by equation (A), glucose present in the test sample converts the enzyme (E_{ox}), such as the oxidized flavin adenine dinucleotide (FAD) center of the enzyme into its reduced form (E_{red}), for example, ($FADH_2$). Because these redox centers are essentially electrically insulated within the enzyme molecule, direct electron transfer to the surface of a conventional electrode does not occur to any measurable degree in the absence of an unacceptably high cell voltage. An improvement to this system involves the use of a nonphysiological redox coupling between the electrode and the enzyme to shuttle electrons between the ($FADH_2$) and the electrode. This is represented by the following scheme in which the redox coupler, typically referred to as a mediator, is represented by M:

$$\text{Glucose} + GO(FAD) \rightarrow \text{gluconolactone} + GO(FADH_2)$$

$$GO(FADH_2) + 2M_{ox} \rightarrow GO(FAD) + 2M_{red} + 2H^+$$

$$2M_{red} \rightarrow 2M_{ox} + 2e^- \text{(at the electrode)}$$

In the scheme, GO(FAD) represents the oxidized form of glucose oxidase and GO(FAD H_2) indicates its reduced form. The mediating species M_{ox}/M_{red} shuttles electrons from the reduced enzyme to the electrode thereby oxidizing the enzyme causing its regeneration in situ.

U.S. Pat. Nos. 5,620,579 and 5,653,863 issued to Genshaw et al., and assigned to the present assignee, disclose apparatus and method for determining the concentration of an analyte in a fluid test sample by applying the fluid test sample to the surface of a working electrode, which is electrochemically connected to a counter electrode, and which surface bears a composition comprising an enzyme specific for the analyte. A mediator is reduced in response to a reaction between the analyte and the enzyme. An oxidizing potential is applied between the electrodes to return at least a portion of the mediator back to its oxidized form before determining the concentration of the analyte to thereby increase the accuracy of the analyte determination. Following this initially applied potential, the circuit is switched to an open circuit or to a potential that substantially reduces the current to minimize the rate of electrochemical potential at the working electrode. A second potential is applied between the electrodes and the current generated in the fluid test sample is measured to determine analyte concentration. Optionally, the accuracy of the analyte determination is further enhanced algorithmically.

SUMMARY OF THE INVENTION

Important aspects of the present invention are to provide a new and improved biosensor for determining the presence or amount of a substance in a sample including an oxidizable species as an internal reference and method of use of the biosensor.

In brief, a biosensor for determining the presence or amount of a substance in a sample and methods of use of the biosensor are provided. The biosensor for receiving a user sample to be analyzed includes a mixture for electrochemical reaction with an analyte. The mixture includes an enzyme, a mediator and an oxidizable species as an internal reference.

The internal reference is defined as the oxidizable species which in one embodiment can be further defined as the reduced form of a reversible redox couple that has an equal or higher redox potential than that of the mediator. The internal reference acts to increase the response current additively for operation potentials that oxidize both species and in the case where glucose is the analyte, a total response current is represented by:

$$I_{total} = I_{int\text{-}ref} + I_{glucose}$$

$$I_{int\text{-}ref} \propto \text{(internal reference) and } I_{glucose} \propto \text{(glucose);}$$

Where $I_{int\text{-}ref}$ is the portion of the total response current due to the internal reference, while $I_{glucose}$ is due to the oxidation of mediator proportional to the glucose concentration.

In accordance with features of the invention, the internal reference can be either the same mediator species or an oxidizable species with a higher redox potential than the mediator. Thus for biosensors with a low operation potential oxidizing only the mediator, the current $I_{int\text{-}ref}$ will be zero. However, for biosensors with a higher operation potential that oxidizes both species, the total response current will be the sum of the portion due to internal reference and that due to glucose. Since the internal reference concentration is fixed, the calibration slope of the sensor will only depend on the sensor response for glucose while the intercept will depend on the added amount of the internal reference. In another

words, the internal reference will only offset the intercept and will not change the calibration slope. Thus, the concept of internal reference provides new and different ways to make glucose biosensors.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention together with the above and other objects and advantages may best be understood from the following detailed description of the preferred embodiments of the invention illustrated in the drawings, wherein:

FIG. **1**A is a block diagram representation of biosensor meter including a biosensor having an internal reference in accordance with the present invention;

FIGS. **1**B, **1**C, and **1**D are diagrams respectively illustrating operational methods for use with the biosensor of FIG. **1** of the invention;

FIGS. **2**A, **2**B, and **2**C are charts showing three cyclic voltammograms of MLB based glucose biosensors with ferrocyanide as the internal reference the biosensor of FIG. **1** of the invention in whole blood samples of 0 mg/dL glucose;

FIG. **3** is a chart illustrating a linear response of the biosensor of FIG. **1** of the invention at different voltage operating potentials;

FIG. **4** is a chart illustrating effect of the added internal reference to the overall voltammetric current using biosensors of FIG. **1** of the invention with 10% printed ferricyanide as the counter electrode;

FIGS. **5**A and **5**B are charts illustrating linear response and increased intercept with increasing internal reference of MLB based biosensors of FIG. **1** of the invention with Ag/AgCl as the counter electrode;

FIGS. **6**A and **6**B are charts illustrating linear response and increased intercept with increasing internal reference of MLB based biosensors of FIG. **1** of the invention with 10% ferricyanide as the counter electrode;

FIG. **7** is a chart illustrating linear relationship of the calibration intercept with increasing internal reference of DEX biosensors of FIG. **1** of the invention with 10% ferricyanide as the counter electrode; and

FIGS. **8**A and **8**B are charts illustrating the ratio of signal to reference results from flow-injection-analysis (FIA) of the residual ferrocyanide from a control reagent ink and the reagent ink with 0.1% ferrocyanide added to the reagent mixture of 20% ferricyanide of a biosensor of FIG. **1** of the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to an electrochemical biosensor for determining the presence or amount of a substance in a sample. The biosensor includes sensor strips containing a working electrode and a counter electrode, each of which is at least partially covered with, for example, a separate reagent layer. The reagent layer on the working electrode includes, for example, an enzyme that interacts with an analyte through an oxidation-reduction reaction and also includes a mediator that is the oxidized form of a redox couple. The biosensor of the invention includes an internal reference or a reduced form of the mediator in the reagent layer on the working electrode. The internal reference is defined as an oxidizable species which in one embodiment can be further defined as a reduced form of a reversible redox couple that has an equal or higher redox potential than that of the mediator. A fixed quantative amount of the internal reference is provided in the reagent layer. The biosensors of the invention including the internal reference or added amount of the reduced form of mediator provide for improvements in that the internal reference acts to anchor the calibration intercept by nature of thermodynamics while maintaining the calibration slope.

Many compounds are useful as mediators due to their ability to accept electrons from the reduced enzyme and transfer them to the electrode. A necessary attribute of a mediator is the ability to remain in the oxidized state under the conditions present on the electrode surface prior to the use of the sensor. Among the more venerable mediators are the oxidized form of organometallic compounds, organic molecules, transition metal coordination complexes. A specific example of mediator is the potassium hexacyanoferrate (III), also known as ferricyanide.

As used in the following specification and claims, the term biosensor means an electrochemical sensor strip or sensor element of an analytical device or an instrument that responds selectively to analytes in an appropriate sample and converts their concentration into an electrical signal. The biosensor generates an electrical signal directly, facilitating a simple instrument design. Also, a biosensor offers the advantage of low material cost since a thin layer of chemicals is deposited on the electrodes and little material is wasted.

The term "sample" is defined as a composition containing an unknown amount of the analyte of interest. Typically, a sample for electrochemical analysis is in liquid form, and preferably the sample is an aqueous mixture. A sample may be a biological sample, such as blood, urine or saliva. A sample may be a derivative of a biological sample, such as an extract, a dilution, a filtrate, or a reconstituted precipitate.

The term "analyte" is defined as a substance in a sample, the presence or amount of which is to be determined. An analyte interacts with the oxidoreductase enzyme present during the analysis, and can be a substrate for the oxidoreductase, a coenzyme, or another substance that affects the interaction between the oxidoreductase and its substrate.

The term "oxidoreductase" is defined as any enzyme that facilitates the oxidation or reduction of a substrate. The term oxidoreductase includes "oxidases," which facilitate oxidation reactions in which molecular oxygen is the electron acceptor; "reductases," which facilitate reduction reactions in which the analyte is reduced and molecular oxygen is not the analyte; and "dehydrogenases," which facilitate oxidation reactions in which molecular oxygen is not the electron acceptor. See, for example, *Oxford Dictionary of Biochemistry and Molecular Biology, Revised Edition*, A. D. Smith, Ed., New York: Oxford University Press (1997) pp. 161, 476, 477, and 560.

The term "oxidation-reduction" reaction is defined as a chemical reaction between two species involving the transfer of at least one electron from one species to the other species. This type of reaction is also referred to as a "redox reaction." The oxidation portion of the reaction involves the loss of at least one electron by one of the species, and the reduction portion involves the addition of at least one electron to the other species. The ionic charge of a species that is oxidized is made more positive by an amount equal to the number of electrons transferred. Likewise, the ionic charge of a species that is reduced is made less positive by an amount equal to the number of electrons transferred.

The term "oxidation number" is defined as the formal ionic charge of a chemical species, such as an atom. A higher oxidation number, such as (III), is more positive, and a lower oxidation number, such as (II), is less positive. A neutral species has an ionic charge of zero. Oxidation of a species

results in an increase in the oxidation number of that species, and reduction of a species results in a decrease in the oxidation number of that species.

The term "redox pair" is defined as two species of a chemical substance having different oxidation numbers. Reduction of the species having the higher oxidation number produces the species having the lower oxidation number. Alternatively, oxidation of the species having the lower oxidation number produces the species having the higher oxidation number.

The term "oxidizable species" is defined as the species of a redox pair having the lower oxidation number, and which is thus capable of being oxidized into the species having the higher oxidation number. Likewise, the term "reducible species" is defined as the species of a redox pair having the higher oxidation number, and which is thus capable of being reduced into the species having the lower oxidation number.

The term "organotransition metal complex," also referred to as "OTM complex," is defined as a complex where a transition metal is bonded to at least one carbon atom through a sigma bond (formal charge of −1 on the carbon atom sigma bonded to the transition metal) or a pi bond (formal charge of 0 on the carbon atoms pi bonded to the transition metal). For example, ferrocene is an OTM complex with two cyclopentadienyl (Cp) rings, each bonded through its five carbon atoms to an iron center by two pi bonds and one sigma bond. Another example of an OTM complex is ferricyanide (III) and its reduced ferrocyanide (II) counterpart, where six cyano ligands (formal charge of −1 on each of the 6 ligands) are sigma bonded to an iron center through the carbon atoms of the cyano groups.

The term "coordination complex" is defined as a complex having well-defined coordination geometry, such as octahedral or square planar geometry. Unlike OTM complexes, which are defined by their bonding, coordination complexes are defined by their geometry. Thus, coordination complexes may be OTM complexes (such as the previously mentioned ferricyanide), or complexes where non-metal atoms other than carbon, such as heteroatoms including nitrogen, sulfur, oxygen, and phosphorous, are datively bonded to the transition metal center. For example, ruthenium hexaamine, or hexaaminoruthenate (II)/(III), is a coordination complex having a well-defined octahedral geometry where six NH_3 ligands (formal charge of 0 on each of the 6 ligands) are datively bonded to the ruthenium center. Ferricyanide is also an example of the coordination complex that has the octahedral geometry. A more complete discussion of organotransition metal complexes, coordination complexes, and transition metal bonding may be found in Collman et al., *Principles and Applications of Organotransition Metal Chemistry* (1987) and Miessler & Tarr, *Inorganic Chemistry* (1991).

The term "mediator" is defined as a substance that can be oxidized or reduced and that can transfer one or more electrons between a first substance and a second substance. A mediator is a reagent in an electrochemical analysis and is not the analyte of interest. In a simplistic system, the mediator undergoes a redox reaction with the oxidoreductase after the oxidoreductase has been reduced or oxidized through its contact with an appropriate substrate. This oxidized or reduced mediator then undergoes the opposite reaction at the electrode and is regenerated to its original oxidation number.

The term "electroactive organic molecule" is defined as an organic molecule that does not contain a metal and that is capable of undergoing an oxidation or reduction reaction. Electroactive organic molecules can behave as redox species and as mediators. Examples of electroactive organic molecules include coenzyme pyrroloquinoline quinone (PQQ), benzoquinones and naphthoquinones, N-oxides, nitroso compounds, hydroxylamines, oxines, flavins, phenazines, phenothiazines, indophenols, and indamines.

The term "electrode" is defined as an electrically conductive substance that remains stationary during an electrochemical analysis. Examples of electrode materials include solid metals, metal pastes, conductive carbon, conductive carbon pastes, and conductive polymers.

Having reference now to the drawings, in FIG. **1** there is illustrated a biosensor meter designated as a whole by the reference character **100** of the preferred embodiment and arranged in accordance with principles of the present invention. Biosensor meter **100** includes a biosensor **102** arranged in accordance with principles of the present invention. Biosensor meter **100** includes microprocessor **104** together with an associated memory **106** for storing program and user data. Digital data from the microprocessor **104** is applied to a digital-to-analog (D/A) converter **108**. D/A converter **108** converts the digital data to an analog signal. An amplifier **110** coupled to the D/A converter **108** amplifies the analog signal. The amplified analog signal output of amplifier **110** is applied to the biosensor **102** of the invention. Biosensor **102** is coupled to an amplifier **112**. The amplified sensed signal is applied to an analog-to-digital (A/D) converter **114** that converts the amplified, analog sensor signal to a digital signal. The digital signal is applied to the microprocessor **104**.

Most of the commercially available disposable biosensors used for monitoring blood glucose require the deposition/printing of a mixture of an enzyme and a mediator with some binding agent. For the application of glucose measurement, the mediator is in the oxidized form of a redox couple. Depending on the redox couple, the mediator can be a very strong oxidant, such as ferricyanide, thereby chemically oxidizing the functional groups after mixing with the enzyme and the binding agent. Subsequently, a small amount of the reduced mediator is formed as impurity in the reagent in the processes of ink mixing, storage and printing. Thus, the end result of mixing and printing the reagent ink is the generation of the reduced form of the redox couple, giving rise to the background current. The formation of this reduced form of the mediator and thus the background current may vary from batch to batch. This process-generated reduced form of the mediator, such as ferrocyanide from ferricyanide, can be oxidized in general to minimize the background signal using the algorithm outlined in the U.S. Pat. Nos. 5,620,579 and 5,653,863, to Genshaw et al., and assigned to the present assignee. However, the process-dependent background signal, which is translated into the calibration intercept, can be spread out in a range of values. At the extremes of these diverged values of intercept, analytical accuracy will be suffered because no reasonable calibration intercept can be assigned to accommodate the diverged intercept.

In accordance with features of the invention, a grade of mediator that contains a certain level of the reduced form of the mediator in the reagent is used for decreasing the effect of the strong oxidant. Thermodynamically, the presence of a small amount of the reduced form of the mediator in the ink mixture of enzyme and mediator decreases the driving force for the conversion from the oxidized to the reduced form. This is advantageously accomplished by adding a small fixed amount of the reduced form of the mediator to the oxidized mediator.

Even though background signal will be generated, the algorithm in the U.S. Pat. Nos. 5,620,579 and 5,653,863 will minimize the effect of background to increase the accuracy of the glucose sensor. The above-identified patents disclose a method that reduces the background bias due to oxidizable impurities in an amperometric sensor used for measuring a

specific analyte, such as glucose, in blood. The background current of such a sensor will increase if it is stored over a long period of time or under stress (heat, moisture, etc.) due to the increased presence of reduced mediator or other reduced impurity present in the sensor such as enzyme stabilizers, e.g. glutamate, and surfactants having reducing equivalents. For example, in a ferricyanide based amperometric sensor, the background bias is related to the presence of ferrocyanide (from the reduction of ferricyanide) near the electrode surface. This accumulated ferrocyanide, as opposed to the ferrocyanide produced during use of the sensor (fresh ferrocyanide), is oxidized back to ferricyanide to reduce the background bias it causes and thereby extend the sensor shelf life. To achieve this objective, the method uses an electrochemical approach. The background bias is further reduced when the electrochemical approach is augmented with an algorithmic correction.

The disclosed method involves first applying a positive potential pulse (called the "burn-off" pulse) which precedes the normal potential profile during use of the biosensor. This is typically accomplished by applying a positive potential of from 0.1 to 0.9 volt (preferably 0.3 to 0.7 volt) between the working and reference electrodes of the sensor for a period of from 1 to 15 seconds (preferably 5 to 10 seconds). The burn-off pulse oxidizes the initial ferrocyanide (or other oxidizable impurity), so that the sensor can begin the assay with a clean background. Typically, the background is not perfectly clean since only a portion of the oxidizable impurity is oxidized by the burn-off pulse. This is the case because the chemical layer covers both the working and the counter electrodes. The initial ferrocyanide exists in the chemical layer since it comes from ferricyanide. When sample fluid is applied and the chemical layer re-hydrates, the ferrocyanide near the working electrode is re-oxidized. The rest of the ferrocyanide diffuses into the sample fluid and is mixed with the glucose. That portion of the initial ferrocyanide cannot be re-oxidized without affecting the glucose. The initial ferrocyanide is near the electrode for a very short time (a few seconds) after the fluid test sample is applied. The reason for this is that the chemicals (enzyme and ferricyanide, etc.) are deposited as a thin layer on the working and counter electrodes. The burn-off technique takes advantage of this since a significant amount of the initial ferrocyanide can be burned off without noticeable reduction of the analyte concentration in the fluid test sample most of which does not come into direct contact with the electrode. Experiments have demonstrated that the background bias of a stressed sensor can be reduced by 40% with proper application of the burn-off pulse.

The disclosed method of the U.S. Pat. Nos. 5,620,579 and 5,653,863 advantageously is applied to minimize the effect of background signal to increase the accuracy of the glucose biosensor meter **100** of the preferred embodiment. The subject matter of the above-identified patents is incorporated herein by reference.

In accordance with features of the invention, the added amount of the reduced form of mediator acts to anchor the calibration intercept by nature of thermodynamics while maintaining the calibration slope. In light of the function the reduced form of mediator, for example, ferrocyanide, plays in the glucose sensor, it is referred to as the internal reference.

Examples of electroactive organic molecule mediators are described in U.S. Pat. No. 5,520,786, issued to Bloczynski et al. on May 28, 1996, and assigned to the present assignee. In particular, a disclosed mediator (compound 18 in TABLE 1) comprising 3-phenylimino-3H-phenothiazine referred to herein as MLB-92, has been used to make a glucose biosensor **102** in accordance with features of the invention. The subject matter of the above-identified patent is incorporated herein by reference.

A commercially available biosensor meter and biosensor is manufactured and sold by Bayer Corporation under the trademark Ascensia DEX. The Ascensia DEX biosensor includes generally as pure a form of ferricyanide as possible for the reagent. The Ascensia DEX biosensor has been used to make a glucose biosensor **102** in accordance with features of the invention by adding an adequate amount of ferrocyanide to the pure ferricyanide. Benefits of adding ferrocyanide defining the internal reference of biosensor **102** to the Ascensia DEX reagent ink include an immediate benefit of increasing the intercept without changing slope, anchoring the intercept range, and increasing long-term stability of biosensor during storage.

In accordance with features of the invention, the MLB-92 mediator having a lower redox potential was used to make a glucose biosensor **102** with special properties. With the addition of adequate amounts of the internal reference, ferrocyanide, the new biosensor system can be made to work with two operation potentials: (1) at 400 mV where both the new mediator and the internal reference are oxidized, and (2) at 100 mV where only the new mediator can be oxidized. The significance of this approach is two-fold. First, the glucose biosensor **102** such formulated (new mediator and internal reference) can be operated at a high potential (+400 mV) to produce currents in a range that fits the calibration characteristics of the hardware requirements of the existing instrument. Secondly, since the lower redox potential and thus a lower oxidation power of the mediator will likely to have virtually no conversion of the oxidized form to the reduced form of the mediator, a lower operation potential (0-100 mV) can be applied to the sensor so as to avoid the oxidation of the internal reference. Thus, a new set of calibration characteristics based on the new mediator, most likely with near zero intercept due to the lower oxidation power, will lead to a better analytical precision for glucose measurements. It will also reduce the matrix interference in the whole blood by avoiding the oxidation of some of the known oxidizable species such as uric acid and acetaminophen.

In accordance with features of the invention, another application of the internal reference to glucose sensors **102** is to add adequately large amount of internal reference to the biosensor system to produce a high current response. Using the double steps algorithm with open circuit between them (Bayer U.S. Pat. Nos. 5,620,579 and 5,653,863), the first potential step is set at 400 mV to produce a current that is mostly due to the internal reference signal while the second step is set at a low potential (0-100 mV) to produce a current signal related to the glucose concentration only. The ratio of the first signal, which should be virtually independent of the whole blood hematocrit, to the second signal at low potential can be used to correct for the analytical bias due to hematocrit effect.

In accordance with features of the invention, the internal reference is defined as the oxidizable species which in one embodiment is further defined as the reduced form of a reversible redox couple that has an equal or higher redox potential than that of the mediator. The concept and use of an internal reference are very common in the field of analytical chemistry. However, no example of using an internal reference for biosensors has been suggested in existing patents or literature. In all three scenarios described above, the internal reference acts to increase the response current additively for operation potentials that oxidize both species and with glucose as the analyte; a total response current is represented by:

$$I_{total}=I_{int\text{-}ref}+I_{glucose}$$

$I_{int\text{-}ref} \propto$ (internal reference) and $I_{glucose} \propto$ (glucose);

Where $I_{int-ref}$ is the portion of the total response current due to the internal reference, while $I_{glucose}$ is due to the oxidation of mediator proportional to the glucose concentration.

In accordance with features of the invention, the internal reference can be either the same mediator species or an oxidizable species with a higher redox potential than the mediator. Thus for biosensors with a low operation potential oxidizing only the mediator, the current $I_{int-ref}$ will be zero. However, for biosensors with a higher operation potential that oxidizes both species, the total response current will be the sum of the portion due to internal reference and that due to glucose. Since the internal reference concentration is fixed, the calibration slope of the sensor will only depend on the sensor response for glucose while the intercept will depend on the added amount of the internal reference. In another words, the internal reference will only offset the intercept and will not change the calibration slope. Thus, the concept of internal reference provides new and different ways to make glucose biosensors.

Referring now to FIGS. **1**B, **1**C, and **1**D, there are at least three modes of operation based on the use of internal reference for glucose biosensors **102** of the invention. Potentiostatically, the three of modes of operation are represented in FIGS. **1**B, **1**C, and **1**D. Each of the illustrated modes of operation include a first burnoff pulse, followed by a second wait period or open circuit, and a final third read pulse, each pulse or period having a selected duration, for example, 10 seconds. In the basic and most immediate operation, ferrocyanide is retained in ferricyanide at the concentration of 0.1 to 1% of the total ferricyanide providing the internal reference for glucose biosensors **102** of the invention. This is depicted in FIG. **1**B where both potentials in the first and the third periods are at the same voltage, for example 400 mV. Retaining of a small percentage of ferrocyanide defining the internal reference can be accomplished either by an appropriate purification process of ferricyanide or by adding an adequate amount of ferrocyanide to the pure ferricyanide. The outcome of these retaining processes is to keep deliberately a desirable amount of ferrocyanide in ferricyanide as a special grade of ferricyanide. This is in contrast to the conventional wisdom of having as pure a form of ferricyanide as possible, such as for the DEX reagent, usually ferrocyanide in the order of 0.05% of ferricyanide or less as impurity. The most desirable amount is 0.1% ferrocyanide in the final formulation for DEX sensor, which will lead to the anchoring of the calibration intercept at a narrower range while maintaining the calibration slope for the DEX sensor.

In FIG. **1**C the second mode of operation is shown, where a desirable amount of ferrocyanide (the internal reference) is added to the reagent of enzyme and a mediator with a redox potential lower than that of the internal reference. The biosensor **102** is expected to work under high and low potentials (for example at 400 mV and 100 mV vs. Ag/AgCl) for existing instruments and instruments with a new hardware requirement. This biosensor can be operated in potential programs depicted in FIG. **1**B for existing instruments **100** and FIG. **1**C for new instruments **100**. Examples of the mediator and internal reference combination include the system of MLB-92 and ferrocyanide as well as ruthenium hexaamine and ferrocyanide. The separation of the two redox potentials is large enough so that there will be generally no oxidation of the internal reference species when operated at the low voltage.

In FIG. **1**D the third mode of operation is shown, where a higher but desirable concentration of ferrocyanide is added to the reagent mixture of enzyme and a mediator with a redox potential lower than that of the internal reference. The amount of the internal reference would produce a current equivalent to about 50% to 75% of the full scale in the calibration range preferably. In the operation algorithm, the first potential step is set to oxidize both the mediator and the internal reference (400 mV) while the second potential step for the read pulse is to oxidize the mediator only (0-100 mV). The current in the first potential step of FIG. **1**D will be most pertinent to the internal reference that is immediately next to the electrode and should have virtually no hematocrit effect. The ratio of the current from the second step to that from the first step will provide a correction for the analytical bias due to hematocrit effect.

Experiments have been carried out to show the feasibility of the method of adding internal reference to a mediator system to overcome existing problems or to enhance sensor performance in accordance with the biosensor **102** of the invention.

Referring now to FIGS. **2**A, **2**B, and **2**C, there are shown three cyclic voltammograms illustrating operation of the biosensor **102** of the invention. The illustrated three cyclic voltammograms are for MLB based glucose biosensors **102** with ferrocyanide as the internal reference in whole blood samples of 0 mg/dL glucose.

FIG. **2**A illustrates working electrode vs. ferricyanide counter electrode, FIG. **2**B illustrates working electrode vs. silver (Ag) and silver chloride (AgCl) or Ag/AgCl counter electrode and FIG. **2**C illustrates working electrode vs. MLB-92 counter electrode. Respective peaks labeled **1** and **2** represent the oxidation of the mediator MLB_{red} (reduced form of MLB) and the internal reference ferrocyanide respectively for all three voltammogram plots. The oxidation peak for MLB_{red} shifts along the potential scale as the redox couple on the counter electrode changes from ferricyanide to Ag/AgCl to MLB-92. However, it can be seen that the relative position of the mediator MLB-92 to the internal reference ferrocyanide is the same in all three voltammogram plots of FIGS. **2**A, **2**B, and **2**C.

Referring to FIG. **3**, there shown in FIG. **3** is a chart illustrating a linear response of the biosensor **102** of the invention at different voltage operating potentials. The biosensor **102** is operated at (1) 400 mV potential and (2) 150 mV potential. FIG. **3** illustrates the linear dose response of MLB-92 mediator based biosensor **102** with 20 mM ferrocyanide as the internal reference. Respective lines labeled EXAMPLE 1 and EXAMPLE 2 are from 400 mV and 150 mV operation potentials against Ag/AgCl counter electrode. As shown in FIG. **3**, the biosensor **102** gives virtually the same slope but with different intercepts for operations at 400 mV and 150 mV potentials. This result demonstrates that the internal reference can be selectively oxidized or avoided by the operation potential. Thus, one biosensor **102** can serve for two different meters.

Examples of the biosensor **102** have been prepared systematically showing the increase of intercept with increasing ferrocyanide as the internal reference while the slopes were kept virtually unchanged. Three working electrode reagents were prepared in the following formulations. These three reagents were pin-deposited on to two sensor formats: (1) Ag/AgCl as the counter electrode, (2) 10% printed ferricyanide as the counter electrode.

Formulations	Enzyme, PQQ-GDH	Mediator MLB-92	Internal Reference Ferricyanide	Buffer and binding agent,
1	20 unit/μL	24 mM	0 mM	0.1M NaCl + phosphate, 1% CMC

-continued

Formulations	Enzyme, PQQ-GDH	Mediator MLB-92	Internal Reference Ferricyanide	Buffer and binding agent,
2	20 unit/μL	24 mM	4 mM	0.1M NaCl + phosphate, 1% CMC
3	20 unit/μL	24 mM	8 mM	0.1M NaCl + phosphate, 1% CMC

FIG. **4** illustrates effect of the added internal reference to the overall voltammetric current using biosensors **102** of the invention with 10% printed ferricyanide as the counter electrode. FIG. **4** provides cyclic voltammograms of sensors with ferrocyanide as the internal reference in whole blood samples of 0 mg/L glucose. Voltammograms labeled A, B and C are with formulations 1, 2 and 3 respectively all with a counter electrode of 10% printed ferricyanide.

The effect of the added internal reference to the overall voltammetric current is shown in FIG. **4** using sensors with 10% printed ferricyanide as the counter electrode. The main oxidation/reduction peaks here are centered around −0.38 Volt vs. 10% ferricyanide, which is due to the mediator MLB. The oxidation peak at about 0-50 mV is due to the internal reference of ferrocyanide. While the oxidation peak for the internal reference ferrocyanide increases with the increases of the internal reference concentration from 0 to 4 to 8 mM, the oxidation peak for the mediator is virtually unchanged. Here the concept of internal reference is explained further by the fact that the main oxidation peak of MLB_{red} is unaffected by the presence of the internal reference.

Referring to FIGS. **5**A and **5**B, charts illustrating linear response and increased intercept with increasing internal reference of MLB based biosensors **102** of the invention with Ag/AgCl as the counter electrode are shown. FIG. **5**A illustrates the linear dose response of MLB based biosensors **102** with 0, 4, and 8 mM ferrocyanide, respectively labeled EXAMPLE 1, EXAMPLE 2, and EXAMPLE 3. FIG. **5**B illustrates intercept and slope as a function of added ferrocyanide in the working electrode reagent of the biosensor **102** of the invention. All three sensors used Ag/AgCl as the counter electrode.

Referring also to FIGS. **6**A and **6**B, charts illustrating linear response and increased intercept with increasing internal reference of MLB based biosensors **102** of the invention with 10% ferricyanide as the counter electrode are shown. FIG. **6**A illustrates the linear dose response of MLB based biosensors **102** with 0, 4, and 8 mM ferrocyanide, respectively labeled EXAMPLE 1, EXAMPLE 2, and EXAMPLE 3. FIG. **6**B illustrates intercept and slope as a function of added ferrocyanide in the working electrode reagent of the biosensor **102** of the invention. All three sensors used 10% printed ferricyanide as the counter electrode.

In the dose response experiments, both sensor series with Ag/AgCl counter electrode of FIGS. **5**A and **5**B, and 10% ferricyanide counter electrode of FIGS. **6**A and **6**B show linear response and increased intercept with increasing internal reference. For practical purpose, the slope of the three sensors in FIGS. **5**A and **5**B is unchanged while the intercept increases linearly with the added ferrocyanide. The same linear relationship of intercept with added ferrocyanide and the flat slope trend are repeated in sensor series with the % printed ferricyanide as the counter electrode, as shown in FIGS. **6**A and **6**B.

Experiments have been carried out to show the addition of ferrocyanide to DEX reagent ink, modification of calibration intercept without changing slope in accordance with the biosensor **102** of the invention.

FIG. **7** illustrates linear relationship of the calibration intercept with increasing internal reference of DEX type biosensors **102** of the invention. Five different formulations in a set format labeled BC**7** in FIG. **7** were made with 0, 0.02, 0.04, 0.06 and 0.08% ferrocyanide mixed in the standard DEX reagent for the DEX sensor. The regression slope and intercepts for these five sensors of the BC**7** format are shown in FIG. **7**. Except for sensor with 0.06% ferrocyanide due to the experimental problems, the intercepts of the other four sensors give a nice linear function with respect to the added amount of ferrocyanide as the internal reference. On the other hand, the slopes of all five sensors fall in a flat line indicating that the addition of the internal reference does not change the slope of the DEX type biosensors **102** of the invention.

FIGS. **8**A and **8**B illustrate the ratio of signal to reference results from flow-injection-analysis (FIA) of the residual ferrocyanide from a control reagent ink and the reagent ink with 0.1% ferrocyanide added to the reagent mixture of 20% ferricyanide of a biosensor **102** of the invention. One of the subtle effects of adding the internal reference ferrocyanide to the DEX reagent ink is to decrease the driving force for the conversion of the mediator ferricyanide to ferrocyanide. Thus, ferricyanide becomes the source of the residual current in the DEX sensor. One way of showing this subtle effect is to monitor the increase of the residual current (background current) of the reagent ink with internal reference along with the control reagent ink over a long period of time. Both reagent inks were stored in refrigeration (2-8° C.) over several weeks. FIG. **8** shows the results of FIA of the residual ferrocyanide from both reagent inks. From FIG. **8**, the ratio of signal-to-reference (S/R) represents the relative amount of ferrocyanide from the reagent ink compared to the added ferrocyanide as the reference in FIA. Thus, the higher the value of S/R from the FIA analysis, the higher the ferrocyanide in the reagent inks. It can be seen from FIG. **8**A that the S/R value increase over the period of six weeks for both the control inks and the reagent ink with added ferrocyanide. However, the reagent ink curve with added ferrocyanide has a slower increase of residual current over the period of six weeks compared to control curves. In FIG. **8**B, the S/R response curves from the control inks and the reagent ink with added ferrocyanide are merged together for comparison. To the first order approximation (since the coefficients for the second order terms of both second order polynomials are very small), the rate of residual current increase over six weeks during refrigeration is about 30% ([0.0918−0.0638]/0.0918=30%) smaller for the reagent ink curve with added ferrocyanide than for the control curves. Thus, it may be understood from FIGS. **8**A and **8**B that the rate of the ferricyanide-to-ferrocyanide conversion in reagent ink is decreased substantially by the addition of the internal reference ferrocyanide to the DEX reagent ink in accordance with biosensor **102** of the invention.

While the present invention has been described with reference to the details of the embodiments of the invention shown in the drawings, these details are not intended to limit the scope of the invention as claimed in the appended claims.

What is claimed is:

1. A method of using a biosensor including a mixture of an only one enzyme, a mediator, and an oxidizable species as an internal reference, said oxidizable species being different than the mediator species and having different redox poten-

tials, wherein said mixture is located directly on a surface of at least a portion of a working electrode, said method comprising the steps of:

applying a first voltage potential in a first voltage period;

providing a set delay period;

applying a second voltage potential in a second voltage period following said delay period; and wherein said first voltage potential and said second voltage potential are selectively provided for oxidizing only said mediator or both said mediator and said internal reference.

2. The method of claim **1** wherein the step of applying said first voltage potential in said first voltage period includes the step of applying a selected first voltage potential in said first voltage period for oxidizing said mediator and said internal reference.

3. The method of claim **1** wherein the step of applying said first voltage potential in said first voltage period includes the step of applying a selected first voltage potential in said first voltage period for oxidizing only said mediator.

4. The method of claim **1** wherein the step of applying said second voltage potential in said second voltage period following said delay period includes the step of applying a selected second voltage potential for oxidizing said mediator and said internal reference.

5. The method of claim **1** wherein the step of applying said second voltage potential in said second voltage period following said delay period includes the step of applying a selected second voltage potential for oxidizing only said mediator.

6. The method of claim **1** wherein the steps of applying said first voltage potential and applying said second voltage potential includes the steps of applying a selected voltage potential in a range between 100 mV and 400 mV.

7. The method of claim **1** wherein the steps of applying said first voltage potential and applying said second voltage potential includes the steps of applying a selected first voltage potential in said first voltage period for oxidizing both said mediator and said internal reference; and applying a selected second voltage potential for oxidizing only said mediator.

8. The method of claim **1** wherein the biosensor includes a mediator comprising 3-phenylimino-3H-phenothiazine; and wherein said internal reference comprises ferrocyanide.

9. The method of claim **1** wherein the steps of applying said first voltage potential and applying said second voltage potential includes the steps of applying a selected first and second voltage potential for oxidizing both said mediator and said internal reference; wherein said internal reference effectively anchoring a calibration intercept within a narrow range and said internal reference effectively maintaining a calibration slope for the biosensor.

10. The method of claim **1** wherein said mediator comprises ruthenium hexamine, and wherein said internal reference comprises ferrocyanide.

11. The method of claim **1** wherein said internal reference comprises ferrocyanide.

12. The method of claim **1** wherein said mediator comprises 3-phenylimino-3H-phenothiazine.

13. The method of claim **1** wherein said mediator comprises ruthenium hexaamine.

14. The method of claim **1** wherein the steps of applying said first voltage potential and applying said second voltage potential includes the steps of applying a selected voltage potential in a range between 0 mV and 400 mV.

15. A method of using a biosensor to assist in determining glucose concentration, the method comprising:

providing a biosensor including a reagent, located directly on a surface of at least a portion of a working electrode, the reagent including an only one enzyme, mediator and an oxidizable species as an internal reference, said oxidizable species being different than the mediator species and having different redox potentials;

applying a first voltage potential in a first voltage period;

providing a set delay period; and

applying a second voltage potential in a second voltage period following said delay period,

wherein said first voltage potential is selectively provided for oxidizing only said mediator or both said mediator and said internal reference, and wherein said second voltage potential is selectively provided for oxidizing only said mediator or both said mediator and said internal reference.

16. The method of claim **15** wherein the enzyme includes glucose oxidase.

17. The method of claim **15** wherein enzyme includes glucose dehydrogenase.

18. The method of claim **15** wherein said mediator reference comprises 3-phenylimino-3H-phenothiazine.

19. The method of claim **18** wherein said internal reference is ferrocyanide.

20. The method of claim **15** wherein the steps of applying said first voltage potential and applying said second voltage potential includes the steps of applying a selected voltage potential in a range between 0 mV and 400 mV.

21. A method of using a biosensor to assist in determining an analyte concentration, the method comprising:

providing a biosensor including a reagent, the reagent, located directly on a surface of at least a portion of the working electrode, including only one enzyme, mediator and an oxidizable species as an internal reference;

applying a first voltage potential in a first voltage period;

providing a set delay period; and applying a second voltage potential in a second voltage period following said delay period;

wherein one of said first voltage potential and said second voltage potential oxidizes only said mediator and the other one of said first voltage potential and said second voltage potential oxidizes both said mediator and said internal reference.

22. The method of claim **21** wherein the analyte is glucose.

23. The method of claim **22** wherein the enzyme includes glucose oxidase or glucose dehydrogenase.

24. The method of claim **21** wherein said mediator reference comprises 3-phenylimino-3H-phenothiazine.

25. The method of claim **21** wherein the steps of applying said first voltage potential and applying said second voltage potential includes the steps of applying a selected voltage potential in a range between 100 mV and 400 mV.

26. The method of claim **21** wherein said first voltage potential oxidizes both said mediator and said internal reference and said second voltage potential oxidizes only said internal reference.

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